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PRINCIPAL INVESTIGATOR: Edward W. Gabrielson, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

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Edward W. Gabrielson, M.D.

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Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

EMail: egabriel@jhmi.edu

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INTRODUCTION

The purpose of this study is to develop a diagnostic blood test for breast cancer. The concept is based on the premise that breast cancers shed sufficient quantities of DNA into plasma to be detected using methylation-specific PCR.

During the second year of this study, a small number of serum samples from breast cancer patients were tested for methylation of genes previously determined to be methylated in the cancer tissue. Disappointingly, methylation was detected in only two of these serum samples. Therefore, in the third year of the project, we tested a highly sensitive method for detection of MSP products. Because the genes that we originally used to assay cancers for methylation showed positive results in relatively low percentages of breast cancer cases, we also profiled methylation of a number of additional genes (12 in all) in over 100 breast cancer cases.

BODY

The overall progress of this project is summarized below:

1. Increasing sensitivity for detection of hypermethylation by fluorescent labeling. We tested various approaches to increasing the sensitivity of the methylation specific PCR (MSP) method (1), including increased numbers of PCR cycle, increased amounts of template DNA, and increased levels of primers. None of these approaches increased sensitivity without producing non-specific PCR products.

We also investigated the use of a fluorescent detection system (Gel-Star), which does help in the recognition of relatively weak PCR products. Overall, the use of this fluorescent detection system increases the number of cases scored as methylated (for any particular gene) by an average of about 25%.

2. Profiling the spectrum of methylation in major classes of breast cancer

Previous studies have generally considered methylation of individual genes in breast cancer. We have now evaluated methylation of 12 different genes in 109 cases of breast cancer, including tumors representing ductal, mucinous, and lobular morphological types. Representative MSP results are shown in the figure below for 3 genes: RARβ, cyclin D2 (CD2), and HIN-1 (2-4). A summary of results for all 12 genes is shown in figure 2.

Figure 1: Analysis of methylation in breast cancer samples using MSP and fluorescent detection. A set of breast cancer samples was analyzed for the RARβ, CD2, and HIN-1 genes. These results are included in the summary shown in figure 2.

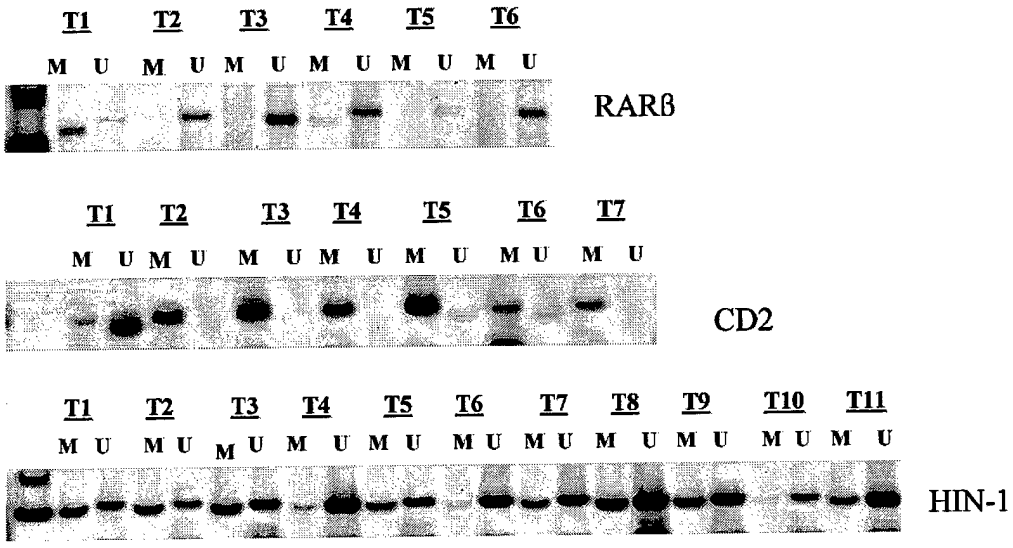
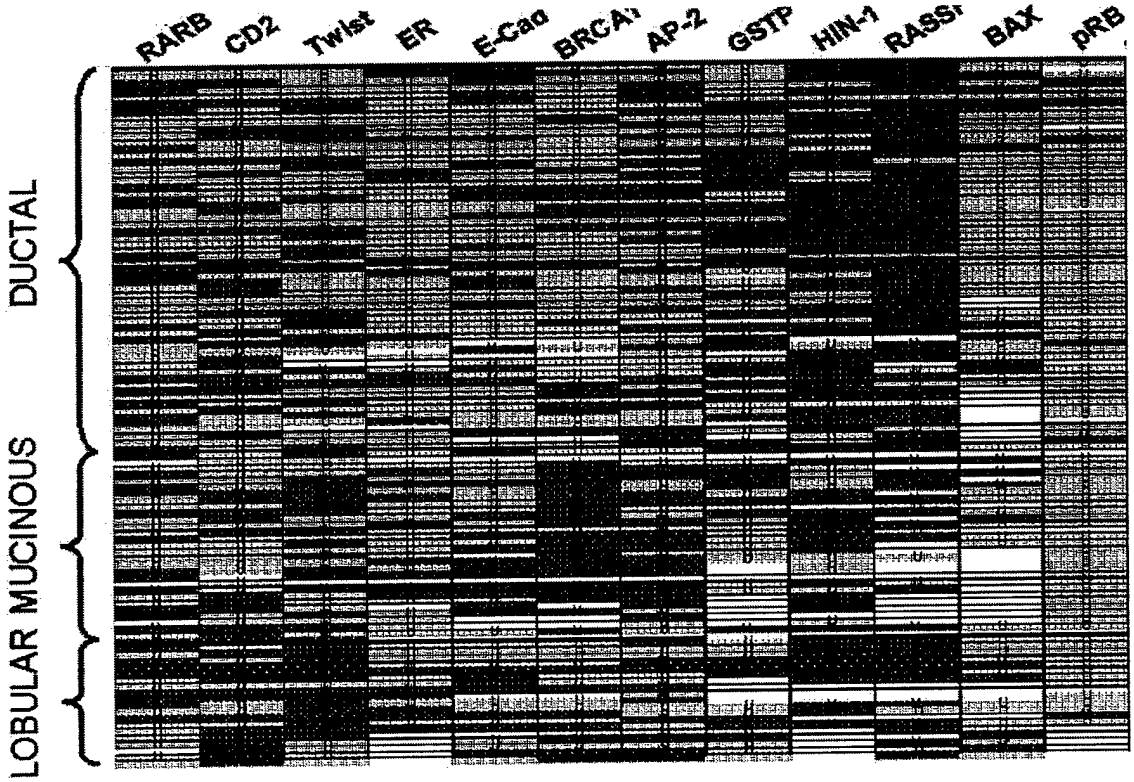


Figure 2: Summary of methylation changes measured in breast cancer samples using MSP and fluorescent detection. A set of breast cancer samples was analyzed for the 12 different genes. Methylation is symbolized by a dark box and absence of methylation (unmethylated) is symbolized by a light tan bar..



It is important to note that >95% of breast cancers have methylation of HIN-1, RASSF1A, or both. The e-cadherin gene, proposed to be a candidate marker in our original application, is much less frequently methylated that these other genes.

We have analyzed this data to determine whether any patterns of methylation correlate with histologic classification. One gene, BrCa1, is methylated at a significantly higher frequency in mucinous cancers than other types of breast cancer, consistent with previous findings (5). Overall, no histologic type of breast cancer has distinctly higher or lower overall levels of methylation, and we find no evidence for a CpG island methylator phenotype of the type reported for colorectal cancer (6).

3. Detection of methylation of marker genes in normal breast and peripheral blood cells.

The utility of measuring methylation for detection of breast cancer is dependant upon the specificity of a positive reaction for the diagnosis of cancer. Therefore, we analyzed normal blood mononuclear cells and normal mammary epithelial cells for methylation using the same high-sensitivity fluorescent detection system used to analyze breast cancer samples. Representative analyses are shown in figure 3 below and the data is summarized in figure 4.

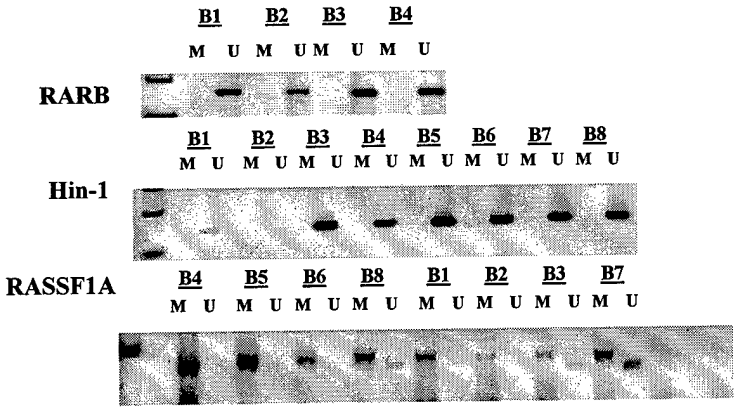


Figure 3: Detection of methylation in normal peripheral blood (B) mononuclear cells by MSP, using fluorescent detection methods. Results are shown for RARβ, HIN-1, and RASSF1A genes.

	Cyclin D2	RARB	E-cadherin	BRCA1	Hin-1	RASSF1A
B1	U	U	U	U	U	
B2				U		U
B3	U	U	U	U		U
B4					U	U
B5					U	U
B6				U		U
B7	U	U	U	U	U	
B8				U	U	U
Methylation rate (%	25	25	25	25	28.6	25

	Cyclin D2	RARB	E-cadherin	BRCA1	ER	RASSF1A
N1	U		U		U	
N2	U		U			
N3	U	U				U
N4	U	U	U		U	U
N5	U	U	U		U	U
ethylation rate ('	0	0	0	100	0	0

Figure 4: Methylation of genes in normal blood (B) mononuclear cells and normal mammary epithelial cells (N).

KEY RESEARCH ACCOMPLISHMENTS

- Methods enhanced for increased detection of methylation.
- Methylation patterns of breast cancer profiled for 12 genes.
- Genes with most frequent methylation identified.
- Non-specific methylation of normal blood cells and normal mammary cells found.

REPORTABLE OUTCOMES

This work has been presented as a poster and platform presentation at the DOD Era of Hope Meeting in Orlando, Florida, September 2002. A manuscript describing the profile of methylation in breast cancer is in final stages of preparation and will be submitted this year. Another manuscript, describing methylation of genes in normal mammary cells, is also in preparation.

CONCLUSIONS

Our finding of methylation in normal cells does temper our optimism for using this approach to develop a blood test to detect breast cancer. We are exploring quantitative methods to set thresholds for determining that levels of methylation are greater than "background", but the finding of any methylation in normal tissues suggests that there will be limits on the sensitivity of this approach.

This project is being continued beyond the original 3-year funding period because the research fellow dedicated to the project left the laboratory prior to completing the project. Funds from the third year of funding are available for this continuation. A set of peripheral blood samples have been collected for completion of this project and these samples will be analyzed after further refinement of methods.

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